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On the use of aptamer microarrays as a platform for the exploration of human prothrombin/thrombin conversion

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ABSTRACT

Microarrays are particular biosensors with multiple grafted probes that are generally used for parallel and simultaneous detection of various targets. In this study, we used microarrays with aptamer probes in order to follow up the different biomolecular interactions of a single enzyme, the thrombin protein, involved in the complex coagulation cascade. More precisely, thanks to label-free surface plasmon resonance imaging, we were able to monitor in real time an important step in the firing of the coagulation cascade in situ—the enzymatic transformation of prothrombin into thrombin, catalyzed by factor Xa. We were also able to appraise the influence of other biochemical factors and their corresponding inhibiting or enhancing behaviors on thrombin activation. Our study opens the door for the development of a complete microarray-based platform not only for the whole coagulation cascade analysis but also for novel drug screening assays in pharmacology.

Microarrays are characterized by the grafting of multiple probes on the same sensor surface, allowing the detection of molecular interactions with different targets in parallel [1,2]. However, microarrays could also be useful for the interrogation of various binding sites on the same target through the use of immobilized probes with different affinities [3], which was the aim of this study. During recent years, aptamers have appeared as a new kind of probe in biosensor technologies [4–6]. These probes are selected in vitro with high affinity for numerous targets as different as simple ions (e.g., Hg^{2+}, Pb^{2+}), small molecules (e.g., adenosine, cocaine), proteins (e.g., thrombin), or even cells and tissues [7–9]. Due to their nucleic acid nature, DNA aptamers present high chemical and thermal stability, easiness for large-scale synthesis, and easiness for chemical modifications useful for labeling and surface grafting.

In the case of protein targets, the antithrombin aptamers are a "gold standard" for the validation of biosensor strategies aiming to reduce the detection limit [7–11]. Indeed, due to the available characterization of two aptamers recognizing two different binding sites of thrombin, numerous strategies with and without sandwich structures have been tested so far [12]. Besides this, the detection of the thrombin protein presents an interest for medical diagnostics due to its implication in the initiation of the coagulation cascade [13]. In particular, these antithrombin aptamers have been shown to display anticoagulation behaviors [14]. In this respect, a microarray platform enabling the real-time follow-up of the coagulation cascade would be highly beneficial for the drug design of pro- or anticoagulant agents in the pharmacology industry. Our current study aimed at crossing the first barriers in this direction by using both antithrombin aptamers as probes for accessing the molecular interactions between thrombin and its natural cofactors in the coagulation cascade. For this prospect, and in order to have access to the kinetics of the molecular interactions without perturbing them, we used the surface plasmon resonance imaging (SPRi)[1] detection technique for its label-free and real-time advantages [12,15,16]. In this article, we demonstrate that a microarray with both antithrombin aptamers present as probes enables the binding studies of thrombin with its known cofactors. More important, we show that we are able to follow the enzymatic biochemical activation of prothrombin into thrombin *in real time.* Such insights give access to the potential inhibiting effects of the partners involved in this important

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[1] *Abbreviations used:* SPRi, surface plasmon resonance imaging; THR, α-thrombin; proTHR, prothrombin; FXa, factor Xa; FVa, factor Va; ATIII, antithrombin; PL, phospholipid–TGT emulsion; BSA, bovine serum albumin; cyt *c*, cytochrome *c*; hep, heparin sodium; NHS, *N*-hydroxysuccinimide; PEG, polyethylene glycol.

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mechanism of the coagulation cascade. In general terms, we demonstrate that instead of using multiple probes on microarrays for the detection of different targets, it is possible to use them to interrogate the multiple molecular interactions of a single target such as thrombin. Based on this first proof-of-principle of the enzymatic transformation of prothrombin into thrombin monitored in real time on a dedicated microarray, we expect that this original strategy may be generalized to the whole coagulation cascade processes based on adequately selected probes.

Materials and methods

Reagents and chemicals

Human α-thrombin (THR), human prothrombin (proTHR), human factor Xa (FXa), human factor Va (FVa), human antithrombin (ATIII), and phospholipid–TGT emulsion (PL, i.e., phospholipid emulsion containing a mixture of highly purified phosphatidyl choline, phosphatidyl serine, and sphingomyelin in Tris–HCl, pH 7.6) were purchased from Cryopep (France). Bovine serum albumin (BSA), cytochrome *c* (cyt *c*), heparin sodium (hep) from porcine intestinal mucosa (MW = 17–19 kDa), 11-mercapto-undecanoic acid, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethylformamide (DMF), and all of the reagents for buffers were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). CH_3O-PEG-SH (MW = 2 kDa) was purchased from Rapp Polymere (Germany). The buffer used for DNA aptamer spotting was a 1 M HK_2PO_4 solution (pH 9.25), whereas the one used for protein dilution, called running buffer, was 20 mM Tris–HCl, 1 mM $MgCl_2$, 120 mM NaCl, 10 mM KCl, and 2 mM $CaCl_2$ (pH 7.4). All chemicals were used without further purification. All oligonucleotides were purchased from Eurogentec (Paris, France) with a primary amine modification at the 5′ position (5′-amino modifier C6) and a 10-thymine spacer on the 5′ end of each sequence. Those sequences are listed in Table 1.

Aptamer microarray fabrication

Oligonucleotides were grafted on gold-coated prisms (Horiba Scientific, Palaiseau, France) as self-assembled monolayers (SAMs). First, a thiol functionality was introduced on the oligonucleotides by conjugation with an activated NHS ester following a protocol described by Daniel and coworkers [12]. Both aptamers (APT1 and APT2) were grafted along with a positive control used to assess the proper chemical grafting and ability for hybridization of microarrayed oligonucleotides (see Table 1 for sequences). The bare gold layer may serve as negative control for nonspecific adsorption. For the grafting, we reproduced a previously described protocol [12] using a piezoelectric dispensing system to depose droplets of solutions containing micromolar concentrations of oligonucleotide and polyethylene glycol (PEG), both modified by thiols. The PEG molecules were used to reduce the nonspecific binding of proteins and to space the aptamers on the surface to increase accessibility. After overnight drying on the bench, prisms were thoroughly rinsed with deionized water and dried under an argon stream for a few seconds. The size of the spots was approximately 500 μm, and the density of probes ranged from 5 to 10 pmol cm^{-2} [11].

Table 1
Oligonucleotide sequences.

Sequence name	Sequence
APT1	5′-NH_2-T_{10}-GGT-TGG-TGT-GGT-TGG-3′
APT2	5′-NH_2-T_{10}-AGT-CCG-TGG-TAG-GGC-AGG-TTG-GGG-TGA-CT-3′
Control	5′-NH_2-T_{10}-TGC-GAT-CGC-AGC-GGT-AAC-CTG-ACC-3′

SPR imaging setup

The detection of thrombin was monitored with an SPR imager (Fig. 1B) apparatus (SPRi-Lab$^+$, Horiba Scientific–GenOptics) equipped with an incoherent light source (λ = 635 nm). The reaction chamber consisted of a hexagonal shape reactor 100 μm high, 15 mm long, and 9 mm wide that was machined in a PEEK flow cell (~15 μl volume). The flow cell was connected to PEEK tubing coupled with a degassing system (Alltech, France) and a syringe Cavro pump (Tecan, USA). The experiments were performed at 25 °C. All injections were performed using a 1-ml injection loop. Prior to the experiment, the microarray was blocked by incubation at room temperature for 90 min in a solution of 500 nM BSA and 500 nM cyt *c*. SPR data were acquired using the software SPRiView (version L3.1.2) furnished by Horiba Scientific–GenOptics. The acquisition of reflectivity signal, monitored with a 12-bit camera (Allied Vision Technologies, Stadtroda, Germany), started once the baseline was stabilized. Reflectivity values were averaged for each replicated spot and plotted on time. To obtain the reflectivity shift on binding of biomolecules, reflectivity values measured at initial times were subtracted from the raw data.

Prothrombin and thrombin real-time detection

Series of injections were tested with the aim of studying the kinetic transformation of prothrombin into thrombin by FXa, alone or within the prothrombinase complex (Fig. 1). The one-way flow rate of the running buffer was 50 μl min^{-1}. To assess the signal specificity, a large excess of cyt *c* (500 nM) was used as a blocking agent and, thus, was added to each sample. First, short injections of thrombin and prothrombin (15 min) were carried out at a concentration of 50 nM protein diluted in the running buffer (Fig. 2). To prove the feasibility of the study, systematic controls were carried out by successive short injections of different elements (Fig. 3): a dilution of phospholipids (PL) at 6 μM, a prothrombinase solution (inspired by Kretz et al. [17] and consisting of 6 μM PL, 0.25 nM FXa, and 0.6 nM FVa diluted in running buffer), several dilutions of thrombin and prothrombin at different concentrations, and finally 100 nM prothrombin preincubated for a few minutes in the prothrombinase solution. The following experiments (see Figs. 4–7 in Results and Discussion) were performed with longer injection times. Samples were delivered in an alternative injection mode (15 μl dispensed volume, 10 μl aspirated volume, 50 μl min^{-1} flow rate) performed 2.5 min after the beginning of the injection in order to ensure the complete filling of the reactor and lasted 45 min. To reduce and optimize sample volumes without decreasing the injection times, we used this alternative injection mode where 15 μl of the sample is flowed one way on the chip and then 10 μl is flowed the way back. This mode allows performing 45-min injections instead of 10-min injections of 500 μl sample. Each sample was systematically prepared just before being injected; in each injection series, prothrombin was the last reactant added to the solution loaded on the microarray, exactly 2 min before reaching the SPR chamber and reacting with the probes. Finally, between each protein injection, the microarray was regenerated by injection of a 50-mM NaOH solution for 2 min at a flow rate of 50 μl min^{-1}. Up to 30 injections and regenerations could be performed without significant loss of signal (<10%).

Results and discussion

Discrimination between thrombin and prothrombin in solution

The aim of this study was to observe the enzymatic transformation of prothrombin into thrombin in situ and to analyze the effects